Tissue inhibitor of metalloproteinase-1 mRNA production and protein secretion are induced by interleukin-1β in 3T3-L1 adipocytes

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Abstract

The adipokine tissue inhibitor of metalloproteinase (TIMP)-1 is upregulated when weight is gained and promotes adipose tissue development. In the present study, the effect of insulin resistance-inducing and proinflammatory interleukin (IL)-1β on TIMP-1 gene expression and secretion was investigated in 3T3-L1 adipocytes. Interestingly, protein secretion and mRNA production of TIMP-1 were significantly stimulated by IL-1β. Thus, IL-1β induced TIMP-1 secretion in a dose-dependent manner with maximal 3.5-fold upregulation seen at 0.67 ng/ml IL-1β relative to untreated cells. Furthermore, TIMP-1 mRNA synthesis was significantly stimulated by IL-1β in a dose-dependent fashion with 2.5-fold induction seen at IL-1β concentrations as low as 0.02 ng/ml and maximal 8.1-fold upregulation found at 20 ng/ml effector. Induction of TIMP-1 mRNA was also time dependent with maximal 9.6-fold upregulation detectable after 8 h of IL-1β treatment. Signaling studies suggested that janus kinase 2 is involved in IL-1β-induced TIMP-1 mRNA expression. Taken together, our results demonstrate that the TIMP-1 expression is selectively upregulated by proinflammatory IL-1β, supporting a direct association between insulin resistance, inflammation, and adipose tissue development in obesity.


Introduction

Obesity is characterized by a chronic inflammatory state. Biologically active proteins secreted from adipose tissue, so-called adipokines, are suggested to link obesity with associated disorders including insulin resistance, type II diabetes mellitus (T2DM), and cardiovascular diseases (Fasshauer & Paschke 2003). Recently, interleukin (IL)-1β has been characterized as a novel fat-secreted adipokine with insulin resistance-inducing and proinflammatory properties besides tumor necrosis factor (TNF)-α and IL-6 (Lagathu et al. 2006, Barksby et al. 2007, Jager et al. 2007). Thus, chronic IL-1β treatment impaired insulin-induced glucose transporter (Glut)-4 expression and markedly inhibited its translocation to the plasma membrane through downregulation of insulin receptor substrate-1 (Jager et al. 2007). Furthermore, long-term IL-1α stimulation of 3T3-L1 adipocytes induced expression of suppressor of cytokine signaling-1 and -3 (He et al. 2006), which are well-established inhibitors of insulin signal transduction (Fasshauer et al. 2004). In addition, IL-1β dramatically decreased the production of the insulin-sensitizing adipokine adiponectin in 3T3-L1 adipocytes and in human primary fat cells (Lagathu et al. 2006). Consistent with these findings, Thomas and co-workers have shown that IL-1 receptor-deficient non-obese diabetic mice were partially protected from the development of T2DM (Thomas et al. 2004). Furthermore, blockage of IL-1 signaling with anakinra, a recombinant human IL-1 receptor antagonist, improved glycemic control in patients with T2DM most likely through improved secretory function of β-cells (Larsen et al. 2007).

Taking these studies into consideration, identification and characterization of downstream targets of IL-1β in adipocytes have become a focus of present research since novel pharmacological targets for the treatment of obesity and associated diseases including insulin resistance, T2DM, hypertension, and atherosclerosis might be derived from these studies.

Tissue inhibitor of metalloproteinase (TIMP)-1 is an adipocyte-secreted protein upregulated in obesity that promotes adipose tissue development (Maquoi et al. 2002, Chavey et al. 2003). Our group has recently shown that TIMP-1 is upregulated both by proinflammatory TNFα and IL-6 (Kralisch et al. 2005) and the β-adrenergic agonist isoproterenol in murine adipocytes (Kralisch et al. 2006). However, a potential regulation of TIMP-1 by proinflammatory IL-1β has not been elucidated so far. Therefore, we investigated the effect of IL-1β on TIMP-1 protein secretion and mRNA production in 3T3-L1 adipocytes in the present study.
Materials and Methods

Materials

Reagents for cell culture were purchased from PAA (Pasching, Austria), and oligonucleotides were from MWG-Biotech (Ebersberg, Germany). AG490, dexamethasone, IL-1β, isobutylmethylxanthine, LY294002, parthenolide, and PD98059 were obtained from Calbiochem (Bad Soden, Germany), and insulin from Roche Molecular Biochemicals.

Culture and differentiation of 3T3-L1 cells

3T3-L1 adipocytes (American Type Culture Collection, Rockville, MD, USA) were differentiated as described previously (Kralisch et al. 2005). In brief, confluent preadipocytes were cultured for 3 days in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose (DMEM-H), 10% fetal bovine serum, and antibiotics (culture medium) further supplemented with 1 μM insulin, 0.5 mM isobutylmethylxanthine, and 0.1 μM dexamethasone. After 3 additional days in culture medium with 1 μM insulin and 3–6 days in culture medium, about 95% of the cells had accumulated fat droplets. All stimulations were carried out in DMEM-H without any additions.

Analysis of TIMP-1 protein secretion

Quantification of TIMP-1 protein secretion into 3T3-L1 cell culture supernatants was performed with a commercially available ELISA from RayBiotech Inc. (Norcross, GA, USA) according to the manufacturer’s instructions.

Quantification of mRNA synthesis

The mRNA expression was determined by quantitative real-time RT-PCR in a fluorescent temperature cycler (7000 sequence detection system, Applied Biosystems, Darmstadt, Germany) as described previously (Kralisch et al. 2005). In brief, RNA was isolated from 3T3-L1 adipocytes using TRizol (Invitrogen, Life Technologies, Inc.). After 1 μg RNA was reverse transcribed with standard reagents (Invitrogen, Life Technologies, Inc.), 2 μl of each RT reaction were amplified in a 26 μl PCR. After initial denaturation at 95°C for 10 min, 40 PCR cycles were performed using the following conditions: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The following primers were used: TIMP-1 (accession no. NM_011593) CTATAGTGCTGGCTGTGGTGGTGTTG (sense) and TCTCCTGGCAGGCCGAGCAAGCAAAGT (antisense); TIMP-2 (accession no. NM_011594) GCCCTCCCTCCCTTTACCTC (sense) and GACATCATAATCCAGCAGCCGACAT (antisense); adiponectin (accession no. U37222) AAGGCAAGGGGCCTTCTCTTCTT (sense) and TATGGATAGGGCCAGTGATGTGG (antisense); CCAAT/enhancer-binding protein (C/EBP)-α (accession no. NM_007678) GGGTGCAGCGAGCCGAGATAAAG (sense) and GCCGGCAGCCGTGGCTCCAGTT (antisense); Glut-4 (accession no. NM_009204) GTGGCCTCTGCTGCTGGAAAGC (sense) and GCGGGGCCCCCTGGCTGAAGAAG (antisense); peroxisome proliferator-activated receptor (PPAR) γ (accession no. NM_011146) CGTGAAGCCCATCAGAGCAATC (sense) and TGGGAGCACGGTGAAG (antisense); and 36B4 (accession no. NM007475) AAGGCGTGCTGGCAGGGGT (sense) and CCAGCGGAGCCACGGTTGT (antisense). SYBR Green I fluorescence was monitored after each cycle. Levels of the different genes of interest were quantified using the second derivative maximum method of the ABI Prism 7000 software (Applied Biosystems) determining the crossing points of individual samples. TIMP-1, TIMP-2, adiponectin, C/EBPα, Glut4, and PPARγ mRNAs were expressed relative to 36B4 that has been used as an internal control due to its resistance to hormonal regulation (Laborda 1991). Specific transcripts were confirmed by melting curve profiles (cooling the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR, and the specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Statistical analysis

Results are shown as mean±S.E.M. Differences between various treatments were analyzed by Mann–Whitney U tests with P values <0.01 considered highly significant and <0.05 considered significant.

Results

TIMP-1 mRNA expression is decreased during adipocyte differentiation

We tested whether TIMP-1 mRNA synthesis is modulated during adipocyte differentiation. To this end, TIMP-1 mRNA was quantified in confluent 3T3-L1 preadipocytes (d0), as well as in cells after 3 (d3), 6 (d6), and 9 (d9) days of differentiation induction. TIMP-1 mRNA was highly expressed in preadipocytes (d0) and decreased to 80% at day 3, 40% at day 6 (P<0.05), and 30% at day 9 (P<0.05) of adipocyte differentiation when compared with day 0 (100%) (Fig. 1A). In contrast, the differentiation markers adiponectin (Fig. 1B), C/EBPα (Fig. 1C), Glut4 (Fig. 1D), and PPARγ (Fig. 1E) were significantly upregulated in 3T3-L1 cells on days 6 (d6) and 9 (d9) of the differentiation course.

IL-1β stimulates TIMP-1 protein secretion dose dependently

Differentiated 3T3-L1 cells were treated with various concentrations of IL-1β for 16 h and TIMP-1 secretion into the medium was measured by ELISA. Interestingly, significant 2.7-fold (P<0.05) upregulation of TIMP-1 protein secretion was first seen at effector concentrations as low as 0.2 ng/ml (Fig. 2). Maximal 3.5-fold upregulation


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from 1849 (basal) to 6520 pg/ml was detectable at an IL-1β concentration of 0.67 ng/ml (P<0.05; Fig. 2).

**IL-1β upregulates TIMP-1 mRNA expression in a dose-dependent manner**

Treatment with IL-1β for 16-h-stimulated TIMP-1 gene expression in a dose-dependent manner. Thus, a significant 2-5-fold induction of TIMP-1 mRNA synthesis was detectable at IL-1β concentrations as low as 0.02 ng/ml (P<0.01; Fig. 3). A maximal 8.1-fold increase was found at 20 ng/ml of the cytokine (P<0.01; Fig. 3). Treatment of 3T3-L1 adipocytes with 20 ng/ml IL-1β for 16 h did not significantly affect adipocyte differentiation as assessed by Oil Red O staining (data not shown).

**IL-1β upregulates TIMP-1 but not TIMP-2 mRNA expression in a time-dependent manner**

Treatment with 20 ng/ml IL-1β increased TIMP-1 mRNA also in a time-dependent manner (Fig. 4A). Thus, significant fourfold (P<0.01) upregulation was first seen after 2 h of treatment, maximal more than ninefold (P<0.01) induction was observed after 8 h, and significant stimulation persisted for up to 24 h (P<0.01) (Fig. 4A). In contrast, TIMP-2 mRNA production was not affected by IL-1β treatment of differentiated 3T3-L1 cells (Fig. 4B). Similar to the results obtained in differentiated adipocytes, 20 ng/ml IL-1β significantly induced TIMP-1 mRNA synthesis time dependently in non-differentiated cells with maximal 2.4-fold (P<0.05) stimulation seen after 4 h of treatment (Fig. 4C).

**Signaling molecules mediating the effect of IL-1β on TIMP-1 mRNA expression**

We elucidated which signaling molecules might mediate the effect of IL-1β on TIMP-1 gene expression. To this end,
3T3-L1 adipocytes were pretreated with specific pharmacological inhibitors of janus kinase (Jak) 2 (AG490, 10 μM), nuclear factor (NF)-κB (parthenolide, 50 μM), p44/42 MAP kinase (PD98059, 50 μM), or phosphatidylinositol (PI) 3-kinase (LY294002, 10 μM) for 1 h before IL-1β (20 ng/ml) was added for 16 h. Treatment of 3T3-L1 adipocytes with AG490, parthenolide, PD98059, and LY294002 for 17 h significantly suppressed basal TIMP-1 expression to 34, 45, 61, and 52% of controls (P < 0.01) respectively (Fig. 5). Again, TIMP-1 mRNA synthesis was increased more than eightfold after 16 h of IL-1β treatment (P < 0.01; Fig. 5). IL-1β-induced TIMP-1 mRNA expression was completely blocked by pharmacological inhibition of Jak2 and NFκB to 69 and 63% of untreated controls respectively (P < 0.01; Fig. 5). In addition, inhibition of p44/42 MAP kinase by PD98059 and PI 3-kinase by LY294002 partially reversed IL-1β-induced TIMP-1 mRNA synthesis by almost 75 and 60% respectively (P < 0.01; Fig. 5).

Synergistic effects of TNFα, IL-6, and IL-1β on TIMP-1 mRNA expression

Finally, we tested whether IL-1β, TNFα, and IL-6 might influence TIMP-1 mRNA expression in 3T3-L1 adipocytes synergistically. As shown in Fig. 6, 16-h treatment with IL-1β (20 ng/ml), TNFα (20 ng/ml), and IL-6 (30 ng/ml) induced TIMP-1 mRNA expression ninefold (P < 0.05), fivefold (P < 0.05), and sevenfold (P < 0.05) respectively. Interestingly, TIMP-1 gene expression further increased when 3T3-L1 cells were treated with IL-1β and TNFα, IL-1β and IL-6, or TNFα and IL-6 (Fig. 6). Maximal 85-fold (P < 0.05) induction of TIMP-1 mRNA was found after treatment of 3T3-L1 adipocytes with the three cytokines combined (Fig. 6).

Discussion

In the present study, we demonstrate for the first time that IL-1β induces TIMP-1 gene expression and protein secretion in 3T3-L1 adipocytes. In contrast, TIMP-2 mRNA synthesis is not altered by IL-1β. These results are in accordance with recent findings from our group showing a specific stimulatory effect of IL-1β on TIMP-1 mRNA expression.
During the development of obesity, an extensive reorganization of adipose tissue that involves adipogenesis, angiogenesis, and remodeling of the extracellular matrix is found (Crandall et al. 1997). Here, TIMP-1 appears as an interesting candidate promoting fat accumulation. Thus, addition of recombinant TIMP-1 promotes adipogenic accumulation of lipid droplets in 3T3-L1 adipocytes when compared with control cells (Alexander et al. 2001). Adipocyte differentiation is accelerated in transgenic mice overexpressing TIMP-1 when compared with wild-type animals (Alexander et al. 2001). In addition, the development of obesity is attenuated in TIMP-1-deficient mice on high-fat diet (Lijnen et al. 2003). Furthermore, upregulated TIMP-1 expression is found in mice on high-fat diet when compared with standard-fat diet (Maquoi et al. 2002). Our group has recently demonstrated that TIMP-1 serum levels are an independent predictor of adiposity in humans with highest levels seen in visceral obesity (Kralisch et al. 2007). Furthermore, elevated TIMP-1 serum levels are associated with the presence of carotid artery lesions in dyslipidemic subjects and are related to progression of atherosclerosis (Beaudeux et al. 2003). These findings suggest that TIMP-1 is not only a passively regulated novel adipocyte-secreted factor but might also actively maintain adipocyte tissue mass in states of increased IL-1β serum levels such as obesity, inflammation, and insulin resistance.

Interestingly, IL-1β is also a positive inductor of TIMP-1 mRNA synthesis in undifferentiated adipocytes. These results support the notion that IL-1β is a principal positive regulator of TIMP-1 mRNA expression in adipose tissue where both preadipocytes and adipocytes are present.

We have further elucidated in the present study by which signaling molecules IL-1β upregulates TIMP-1 expression. Binding of IL-1β to the extracellular domain of the IL-1 receptor leads to activation of IL-1 receptor-associated kinase (Cao et al. 1996) which, in turn, stimulates downstream signaling proteins such as NFκB (Dinarello 1997). In the present study, we show that pharmacological inhibition of NFκB by parthenolide reverses IL-1β-induced TIMP-1 mRNA expression suggesting a potential role of this signaling molecule in IL-1β-regulated TIMP-1 mRNA synthesis. However, it has to be pointed out that under the conditions studied, parthenolide significantly decreases cell viability assessed by trypan blue staining when compared with untreated 3T3-L1 adipocytes (data not shown). Therefore, we cannot exclude the possibility that downregulation of TIMP-1 after parthenolide pretreatment is due to a toxic effect of this inhibitor. In contrast, AG490, PD98059, and LY294002 do not influence cell viability (data not shown). Jak2 (Doi et al. 2002), p44/42 MAP kinase (Stylianou & Saklatvala 1998), and PI 3-kinase (Reddy et al. 1997) are downstream signaling molecules of IL-1β which downregulate TIMP-1 synthesis in fat cells since inhibition of this signaling protein leads to a complete reversal of the effects of IL-1β. Pharmacological inhibition of p44/42 MAP kinase and PI 3-kinase downregulates basal TIMP-1 mRNA expression.

Effect of proinflammatory TNFα and IL-6 on TIMP-1 but not TIMP-2 (Kralisch et al. 2005). It is interesting to note in this context that no evidence has been presented so far indicating that TIMP-2 like TIMP-1 influences adipose tissue physiology.

**Figure 5** Signaling molecules involved in IL-1β-induced TIMP-1 mRNA synthesis. After serum starvation, 3T3-L1 adipocytes were cultured in the presence or absence of AG490 (AG, 10 μM), parthenolide (Part, 50 μM), PD98059 (PD, 50 μM), or LY294002 (LY, 10 μM) for 1 h before IL-1β (20 ng/ml) was added for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine TIMP-1 normalized to 36B4 as described in Materials and Methods. Data are expressed relative to non-treated control (Con) cells (= 100%). Results are the means ± S.E.M. of five independent experiments. **P<0.01 comparing non-treated cells with inhibitor- and IL-1β-pretreated cells, as well as comparing IL-1β-treated with inhibitor-pretreated adipocytes.

**Figure 6** IL-1β, TNFα, and IL-6 induce TIMP-1 mRNA synergistically. After serum starvation, 3T3-L1 adipocytes were cultured in the presence or absence of IL-1β (20 ng/ml), TNFα (20 ng/ml), and IL-6 (30 ng/ml) for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine TIMP-1 normalized to 36B4 as described in Materials and Methods. Data are expressed relative to non-treated control cells (= 100%). Results are the means ± S.E.M. of four independent experiments. *P<0.05 comparing effector treated with untreated cells.
synthesis and partially reverses IL-1β-induced TIMP-1 mRNA expression. These data indicate that both signaling molecules are principal positive mediators of TIMP-1 gene expression in adipocytes.

Interestingly, TIMP-1 gene expression further increases when 3T3-L1 cells are treated with IL-1β and TNFα, IL-1β and IL-6, or TNFα and IL-6. Furthermore, maximal induction of TIMP-1 mRNA is found after treatment with the three cytokines combined. These results indicate that the cytokines tested might work as a network with synergistic effects. Since IL-1β significantly stimulates IL-6 mRNA and protein expression (data not shown) and IL-6 is a potent inductor of TIMP-1 synthesis (Kralisch et al. 2005), it is well possible that IL-6 contributes to IL-1β-induced TIMP-1.

Taken together, we demonstrate for the first time that IL-1β is a potent stimulator of TIMP-1 protein secretion and mRNA production in 3T3-L1 adipocytes in vitro. Furthermore, we present evidence that the positive effect of IL-1β is mediated via Jak2.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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